PATENT
454313-2338

PORCINE CIRCOVIRUS AND PARVOVIRUS VACCINE

This application claims priority from French application No. 98 08777, filed July 6, 1998. Reference is also made to U.S. application Serial No. 09/161,092, filed September 25, 1998 as a continuation-in-part of U.S. application Serial No. 09/082,558, filed May 21, 1998, claiming priority from French applications Nos. 97 12382, 98 00873 and 98 03707, filed October 3, 1997, January 22, 1998 and March 20, 1998, respectively. Reference is further made to the U.S. applications of Audonnet et al. and Bublot et al., Serial Nos. 60/138,352 and 60/138,478, respectively, both filed June 10, 1999 ("DNA VACCINE-PCV", and "PORCINE CIRCOVIRUS RECOMBINANT POXVIRUS VACCINE", respectively; attorney dockets 454313-2335 and TH-015, respectively). Reference is additionally made to each of the documents cited in the text and in the record or prosecution of each of the aforementioned U.S. and French applications, including without limitation WO 98/03658, published January 29, 1998 from PCT/FR97/01313, filed July 15, 1997 and designating the U.S. and claiming priority from French application 96 09338, filed July 19, 1996 (the U.S. continuation-in-part of PCT/FR97/01313 being U.S. application Serial No. 09/232,468, filed January 15, 1999). Each of the aforementioned U.S., PCT and French applications (including parenthetically), and each document cited in the text and the record or prosecution of each of the aforementioned U.S., PCT and French applications (including parenthetically), is hereby incorporated herein by reference; and, technology in each of the aforementioned U.S., PCT and French applications (including parenthetically), and each document cited in the text and the record or prosecution of each of the aforementioned U.S., PCT and French applications (including parenthetically) can be used in the practice of this invention.

Furthermore, with respect to equivalent sequences capable of hybridizing under high stringency conditions or having a high homology with nucleic acid molecules employed in the invention, "hybridizing under high stringency conditions" can be synonymous with "stringent hybridization conditions", a term which is well known in the art; see, for example, Sambrook, "Molecular Cloning, A Laboratory Manual" second ed., CSH Press, Cold Spring Harbor, 1989; "Nucleic Acid Hybridisation, A Practical Approach", Hames and Higgins eds., IRL Press, Oxford, 1985; both incorporated herein by reference. With respect to nucleic acid molecules and polypeptides which can be used in the practice of the invention, the nucleic acid molecules and polypeptides advantageously have at least about 84 to 85% or greater homology or identity, such as at least about 85% or about 86% or about 87% or about 88% or about 89% homology or

identity, for instance at least about 90% or homology or identity or greater, such as at least about 91%, or about 92%, or about 93%, or about 94% identity or homology, more advantageously at least about 95% to 99% homology or identity or greater, such as at least about 95% homology or identity or greater e.g., at least about 96%, or about 97%, or about 98%, or about 99%, or even about 100% identity or homology, or from about 84 to about 100% or from about 90 to about 99 · or about 100% or from about 95 to about 99 or about 100% identity or homology, with respect to sequences disclosed or described herein and fragments thereof herein disclosed or described (including subsequences discussed below); and thus, the invention comprehends a vector encoding an epitope or epitopic region of a PCV isolate or a composition comprising such an epitope, compositions comprising an epitope or epitopic region of a PCV isolate, and methods for making and using such vectors and compositions, e.g., the invention also comprehends that these nucleic acid molecules and polypeptides can be used in the same fashion as the herein mentioned nucleic acid molecules, fragments thereof and polypeptides. In this regard, it is noted that homology between PCV1 and PCV2 is about 84% to about 85% and that within the PCV2 group homology is from about 95% to about 99%.

Nucleotide sequence homology can be determined using the "Align" program of Myers and Miller, ("Optimal Alignments in Linear Space", CABIOS *4*, 11-17, 1988, incorporated herein by reference) and available at NCBI. Alternatively or additionally, the term "homology" or "identity", for instance, with respect to a nucleotide or amino acid sequence, can indicate a quantitative measure of homology between two sequences. The percent sequence homology can be calculated as $(N_{ref} - N_{dif})*100/N_{ref}$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence similarity of 75% with the sequence AATCAATC (N_{ref} = 8; N_{dif}=2).

Alternatively or additionally, "homology" or "identity" with respect to sequences can refer to the number of positions with identical nucleotides or amino acids divided by the number of nucleotides or amino acids in the shorter of the two sequences wherein alignment of the two sequences can be determined in accordance with the Wilbur and Lipman algorithm (Wilbur and Lipman, 1983 PNAS USA 80:726, incorporated herein by reference), for instance, using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4, and computer-assisted analysis and interpretation of the sequence data including alignment can be conveniently

performed using commercially available programs (e.g., Intelligenetics ™ Suite, Intelligenetics Inc. CA). When RNA sequences are said to be similar, or have a degree of sequence identity or homology with DNA sequences, thymidine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence (*see also* alignment used in Figures and in Appendix I). RNA sequences within the scope of the invention can be derived from DNA sequences, by thymidine (T) in the DNA sequence being considered equal to uracil (U) in RNA sequences.

Additionally or alternatively, amino acid sequence similarity or identity or homology can be determined using the BlastP program (Altschul *et al.*, Nucl. Acids Res. 25, 3389-3402, incorporated herein by reference) and available at NCBI (used in determining sequence homology, as shown in Appendix I; *see also* the Examples). The following references (each incorporated herein by reference) also provide algorithms for comparing the relative identity or homology of amino acid residues of two proteins, and additionally or alternatively with respect to the foregoing, the teachings in these references can be used for determining percent homology or identity: Needleman SB and Wunsch CD, "A general method applicable to the search for similarities in the amino acid sequences of two proteins," J. Mol. Biol. 48:444-453 (1970); Smith TF and Waterman MS, "Comparison of Bio-sequences," Advances in Applied Mathematics 2:482-489 (1981); Smith TF, Waterman MS and Sadler JR, "Statistical characterization of nucleic acid sequence functional domains," Nucleic Acids Res., 11:2205-2220 (1983); Feng DF and Dolittle RF, "Progressive sequence alignment as a prerequisite to correct phylogenetic trees," J. of Molec. Evol., 25:351-360 (1987); Higgins DG and Sharp PM, "Fast and sensitive multiple sequence alignment on a microcomputer," CABIOS, 5: 151-153 (1989); Thompson JD, Higgins DG and Gibson TJ, "ClusterW: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, positions-specific gap penalties and weight matrix choice, Nucleic Acid Res., 22:4673-480 (1994); and, Devereux J, Haeberlie P and Smithies O, "A comprehensive set of sequence analysis program for the VAX," Nucl. Acids Res., 12: 387-395 (1984).

The disclosed nucleic acid sequences or portions or fragments thereof, e.g., subsequences comprising at least about 12 nucleotides in length, for instance, at least about 15, about 18, about 21, about 24 or about 27 nucleotides in length, such as at least about 30, about 33, about 36, about 39 or about 42 nucleotides in length, for example, a nucleic acid molecule of at least about 12 nucleotides in length such as about 12 to about 30, about 12 to about 50 or about 12 to about 60, or about 12 to about 75 or about 12 to about 100 or more nucleotides in length may be useful in

hybridization, e.g., as probes or primers; and, the invention further comprehends vectors or plasmids containing and/or expressing such a nucleic acid molecule, as such as a nucleic acid molecule can encode an epitope or an epitopic region or a polypeptide which is functionally equivalent to polypeptides expressed by herein mentioned sequences, well as uses of such nucleic acid molecules, e.g., for expression thereof either *in vitro* or *in vivo*, or for amplifying or detecting a herein defined gene or a homolog thereof, and the use of such vectors, e.g., in inventive compositions.

The nucleic acids used for hybridization can, of course, be conveniently labeled by incorporating or attaching, e.g., a radioactive or other marker. Such markers are well known in the art. The labeling of said nucleic acid molecules can be effected by conventional methods. The presence or expression of PCV or genes thereof can be monitored by using a primer pair that specifically hybridizes to either of the corresponding nucleic acid sequences and by carrying out a PCR reaction according to standard procedures. Specific hybridization of the above mentioned probes or primers preferably occurs at stringent hybridization conditions. A probe or primer can be any stretch of at least 8, preferably at least 10, more preferably at least 12, 13, 14, or 15, such as at least 20, e.g., at least 23 or 25, for instance at least 27 or 30 nucleotides in a herein defined nucleic acid molecule which are unique thereto; and, advantageously the probe or primer encodes an epitope or epitopic region, such that the probe or primer can also be useful for expression of an antigenic or immunogenic polypeptide whereby the polypeptide or a vector expressing it are useful in inventive compositions. As to PCR or hybridization primers or probes and optimal lengths therefor, reference is also made to Kajimura et al., GATA 7(4):71-79 (1990), incorporated herein by reference. Furthermore, expression of PCV nucleic acid molecules are useful in generating antibodies, which antibodies can be used to detect the presence or absence of PCV (or antigens thereof) in a sample or specimen; or, the expressed polypeptides can be used to detect the presence or absence of antibodies to PCV in a sample or specimen. Thus, nucleic acid molecules and expression products thereof have diagnostic utilities too.

The invention is discussed in more detail in the following text.

The present invention relates to a vaccine against the PMWS syndrome (*Porcine Multisystemic Wasting Syndrome* also called *Post-Weaning Multisystemic Wasting Syndrome*).

Various documents are cited in the following text, and various documents are referenced or cited in documents cited in the following text. There is no admission that any of these documents are indeed prior art as to the present invention. All documents cited herein and all documents referenced or cited in documents cited herein are hereby incorporated herein by reference.

PCV (for "Porcine CircoVirus") was originally detected as a noncytopathogenic contaminant in pig kidney cell lines PK/15. This virus was classified among the Circoviridae with the chicken anaemia virus (CAV for *Chicken Anaemia Virus*) and the PBFDV virus (*Pscittacine Beak and Feather Disease Virus*). It is a small nonenveloped virus (from 15 to 24 nm) whose common characteristic is to contain a genome in the form of a circular single-stranded DNA of 1.76 to 2.31 kb. It was first thought that this genome encoded a polypeptide of about 30 kDa (Todd et al., Arch Virol 1991, 117; 129-135). Recent work has however shown a more complex transcription (Meehan B. M. et al., 1997, 78; 221-227). Moreover, no significant homologies in nucleotide sequence or in common antigenic determinants are known between the three types of circoviruses known.

The PCV derived from the PK/15 cells is considered not to be pathogenic. Its sequence is known from B.M. Meehan et al., J. Gen. Virol 1997 (78) 221-227. It is only very recently that some authors have thought that strains of PCV could be pathogenic and associated with the PMWS syndrome (Gupi P.S. Nayar et al., Can. Vet. J, vol. 38, 1997: 385-387 and Clark E.G., Proc. Am. Assoc. Swine Prac. 1997; 499-501). Nayar et al. have detected PCV DNA in pigs having the PMWS syndrome using PCR techniques.

The PMWS syndrome detected in Canada, the United States and France is clinically characterized by a gradual loss of weight and by manifestations such as tachypnea, dyspnea and jaundice. From the pathological point of view, it is manifested by lymphocytic or granulomatous infiltrations, lymphadenopathies and, more rarely, by hepatitis and lymphocytic or granulomatous nephritis (Clark E.G., Proc. Am. Assoc. Swine Prac. 1997; 499-501; La Semaine Vétérinaire No. 26, supplement to La Semaine Vétérinaire 1996 (834); La Semaine Vétérinaire 1997 (857): 54; Gupi P.S. Nayar et al., Can. Vet. J, vol. 38, 1997; 385-387).

The applicant has succeeded in isolating five new PCV strains from pulmonary or ganglionic samples obtained from farms situated in Canada, the United States (California) and France (Brittany). These viruses have been detected in lesions in pigs with the PMWS syndrome, but not in healthy pigs.

The applicant has, in addition, sequenced the genome of four of these strains, namely the strains obtained from Canada and the United States as well as two French strains. The strains exhibit a very strong homology with each other at the nucleotide level, exceeding 96% and much weaker with the PK/15 strain, about 76%. The new strains can thus be considered as being representative of a new type of porcine circovirus, called here type II, type I being represented by PK/15.

Purified preparations of five strains were deposited under the Budapest Treaty at the ECACC (European Collection of Cell Cultures, Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom) on Thursday 2 October 1997:

- accession No. V97100219 (called here Imp. 1008PCV)
- accession No. V9700218 (called here Imp. 1010PCV)
- accession No. V97100217 (called here Imp. 999PCV),

and, on Friday 16 January 1998:

- accession No. V98011608 (called here Imp. 1011-48285)

- accession No. V98011609 (called here Imp. 1011-48121).

The applicant has observed that, in a trial for experimental reproduction of the porcine multisystemic wasting syndrome, a porcine parvovirus combined with the porcine circovirus could lead to a worsening of the disease.

The subject of the present invention is therefore a vaccination of pigs using a porcine circovirus, in particular type I or type II, preferably type II, vaccine, combined with a vaccination with a porcine parvovirus vaccine. This is understood to mean vaccination with either a bivalent vaccine, or the simultaneous use, in pigs, of a porcine circovirus vaccine and of a porcine parvovirus vaccine.

The reference parvovirus strain is the NADL-2 strain which is accessible from the ATCC collection under the reference VR-742. Vaccination against the porcine parvovirus is well known to persons skilled in the art and vaccines against the porcine parvovirus are commercially available. There may be mentioned by way of example: Parvovax® (inactivated vaccine against porcine parvovirosis, distributed by MERIAL). See also e.g. P. Vannier et A. Laval., Point. Vét. 1993, 25 (151),53-60 ; G. Florent et al., Proceedings of the Ninth Congress of Pig Veterinary Society, July 15-18, 1986, Barcelona, Spain. For DNA vaccines, one can refer e.g. to WO-A-98 03658.

The subject of the present invention is therefore an antigenic preparation directed against the PMWS syndrome, comprising at least one porcine circovirus antigen (preferably type II circovirus) and at least one porcine parvovirus antigen. In accordance with the invention, the porcine circovirus antigen (preferably type II circovirus) and the porcine parvovirus antigen comprise, independently of each other, an antigen chosen from the group consisting of an attenuated live whole antigen, an inactivated whole antigen, a subunit antigen, a recombinant live vector and a DNA vector. It

is understood that the combination according to the invention may involve the use of any appropriate antigen or antigenic preparation form, it being understood that it is not necessary to use the same form for a given combination. The antigenic preparation may comprise, in addition, as is known per se, a vehicle or excipient acceptable from the veterinary point of view, and optionally an adjuvant acceptable from the veterinary point of view.

The subject of the present invention is also an immunogenic composition or a vaccine against the PMWS syndrome, comprising an effective quantity of circovirus + parvovirus antigenic preparation as described above, in a vehicle or excipient acceptable from the veterinary point of view, and optionally an adjuvant acceptable from the veterinary point of view. An immunogenic composition elicits an immunological response which can, but need not be, protective. A vaccine composition elicits a protective response. Accordingly, the term "immunogenic composition" include a vaccine composition" (as the former term can be protective composition).

The subject of the invention is also an immunological or a vaccination kit containing, packaged separately, an antigenic preparation or an immunogenic composition or a vaccine against the porcine circovirus and an antigenic preparation or an immunogenic composition or a vaccine against the porcine parvovirus. This kit may have the various characteristics set out above for the antigenic preparations, immunogenic compositions and vaccines.

The subject of the invention is also a method of immunization or of vaccination against the PMWS syndrome, comprising the administration of an immunogenic composition or a vaccine against the porcine circovirus and of an immunogenic composition or a vaccine against the porcine parvovirus or the administration of a bivalent immunogenic composition or vaccine, comprising, in the same formulation, an

antigenic preparation specific to each virus. This method of immunisation or vaccination uses in particular the vaccines as defined above.

The subject of the invention is also the use of an antigenic preparation or of an immunogenic composition or a vaccine against the parvovirus, as in particular defined supra, for the preparation of a pharmaceutical composition intended to be used in the context of the prevention of the PMWS syndrome, in combination with an antigenic preparation or an immunogenic composition or a vaccine against the porcine circovirus.

For the production of circovirus antigenic preparations, the circoviruses may be obtained after passage on cells, in particular cell lines, e.g. PK/15 cells. The culture supernatants or extracts, optionally purified by standard techniques, may be used as antigenic preparation.

In the context of attenuated antigenic preparations and attenuated immunogenic compositions or vaccines, the attenuation may be carried out according to the customary methods, e.g. by passage on cells, preferably by passage on pig cells, especially cell lines, such as PK/15 cells (for example from 50 to 150, especially of the order of 100, passages). These immunogenic compositions and vaccines comprise in general a vehicle or diluent acceptable from the veterinary point of view, optionally an adjuvant acceptable from the veterinary point of view, as well as optionally a freeze-drying stabilizer.

These antigenic preparations, immunogenic compositions and vaccines will preferably comprise from 10^3 to 10^7 TCID50 of the attenuated virus in question.

They may be antigenic preparations, immunogenic compositions and vaccines based on inactivated whole antigen. The inactivated immunogenic compositions and vaccines comprise, in addition, a vehicle or a diluent acceptable from the veterinary point of view, with

optionally in addition an adjuvant acceptable from the veterinary point of view.

The circoviruses according to the invention, with the fractions which may be present, are inactivated according to techniques known to persons skilled in the art. The inactivation will be preferably carried out by the chemical route, e.g. by exposing the antigen to a chemical agent such as formaldehyde (formalin), paraformaldehyde, β-propiolactone or ethyleneimine or its derivatives. The preferred method of inactivation will be herein the exposure to a chemical agent and in particular to ethyleneimine or to β-propiolactone.

Preferably, the inactivated antigenic preparations and the inactivated immunogenic compositions and vaccines according to the invention will be supplemented with adjuvant, advantageously by being provided in the form of emulsions, for example water-in-oil or oil-in-water, according to techniques well known to persons skilled in the art. It will be possible for the adjuvant character to also come from the incorporation of a customary adjuvant compound into the active ingredient.

Among the adjuvants which may be used, there may be mentioned by way of example aluminium hydroxide, the saponines (e.g. Quillaja saponin or Quil A; see Vaccine Design, The Subunit and Adjuvant Approach, 1995, edited by Michael F. Powel and Mark J. Newman, Plennum Press, New-York and London, p.210), Avridine® (Vaccine Design p. 148), DDA (Dimethyldioctadecyl-ammonium bromide, Vaccine Design p. 157), Polyphosphazene (Vaccine Design p. 204), or alternatively oil-in-water emulsions based on mineral oil, squalene (e.g. SPT emulsion, Vaccine Design p. 147), squalene (e.g. MF59, Vaccine Design p. 183), or water-in-oil emulsions based on metabolizable oil (preferably according to WO-A-94 20071) as well as the emulsions described in US-A-5,422,109. It is also

possible to choose combinations of adjuvants, for example Avridine® or DDA combined with an emulsion.

These antigenic preparations, immunogenic compositions and vaccines will preferably comprise from 10^5 to 10^8 TCID50 of the inactivated whole virus in question.

The adjuvants for live vaccines described above can be selected from those given for the inactivated. The emulsions are preferred. To those indicated for the inactivated vaccine, there may be added those described in WO-A-9416681.

As freeze-drying stabilizer, there may be mentioned by way of example SPGA (Bovarnik et al., J. Bacteriology 59, 509, 950), carbohydrates such as sorbitol, mannitol, starch, sucrose, dextran or glucose, proteins such as albumin or casein, derivatives of these compounds, or buffers such as alkali metal phosphates.

The antigenic preparations, immunogenic compositions and vaccines according to the invention may comprise one or more active ingredients (antigens) of one or more circoviruses and/or parvoviruses according to the invention.

The applicant has, in addition, obtained the genome of four of the type II porcine circovirus isolates, identified SEQ ID NO: 1 to 4. The sequence of strain PK-15 is given as SEQ ID NO: 5. It goes without saying that the invention automatically covers the equivalent sequences, that is to say the sequences which do not change the functionality or the strain-specificity of the sequence described or of the polypeptides encoded by this sequence. There will of course be included the sequences differing by degeneracy of the code.

The invention also covers the equivalent sequences in the sense that they are capable of hybridizing with the above sequence under high stringency conditions and/or have a high homology with the strains of the invention.

These sequences and their fragments can be advantageously used for the in vitro or in vivo expression of polypeptides with the aid of appropriate vectors.

In particular, the open reading frames (ORF1-13), forming DNA fragments according to the invention, which can be used to this effect have been identified on the genomic sequence of the type II circoviruses. The invention relates to any polypeptide containing at least one of these open reading frames (corresponding amino acid sequence). Preferably, the invention relates to a protein essentially consisting of ORF4, ORF7, ORF10 or ORF13.

For the expression of subunits in vitro, as a means of expression, E. coli or a baculovirus will be preferably used (US-A-4,745,051). The coding sequence(s) or their fragments may be integrated into the baculovirus genome (e.g. the baculovirus Autographa californica Nuclear Polyhedrosis Virus AcNPV) and the latter can be then propagated on insect cells, e.g. Spodoptera frugiperda Sf9 (deposit ATCC CRL 1711). The subunits can also be produced in eukaryotic cells such as yeasts (e.g. Saccharomyces cerevisiae) or mammalian cells (e.g. CHO, BHK).

The subject of the invention is also the use as subunits of the polypeptides which will be produced in vitro by these expression means, and then optionally purified according to conventional techniques. The subunit immunogenic compositions and vaccines comprise at least one polypeptide as thus obtained, or fragment, in a vehicle or diluent acceptable from the veterinary point of view and optionally an adjuvant acceptable from the veterinary point of view.

For the expression in vivo for the purpose of producing immunogenic compositions and vaccines of the recombinant live type or DNA type, the coding sequence(s) or their fragments are inserted into an appropriate expression vector under conditions allowing the expression of the polypeptide(s). As appropriate

live vectors, there may be used preferably live viruses, preferably capable of multiplying in pigs, nonpathogenic for pigs (naturally nonpathogenic or rendered as such), according to techniques well known to persons skilled in the art. There may be used in particular pig herpesviruses such as Aujeszky's disease virus, porcine adenovirus, poxviruses, especially vaccinia virus, avipox virus, canarypox virus, swinepox virus. DNA vectors can also be used as vectors (WO-A-9011092,WO-A-9319813,WO-A-9421797, WO-A-9520660).

The subject of the invention is therefore also the vectors and the recombinant live type or DNA (polynucleotide) type immunogenic compositions or vaccines thus prepared, their preparation and their use, the immunogenic compositions and the vaccines comprising, in addition, a vehicle or diluent acceptable from the veterinary point of view.

By definition, a DNA immunogenic composition or vaccine comprises a DNA vector which is a circular vaccinal plasmid, supercoiled or otherwise, or a linear DNA molecule, incorporating and expressing in vivo a nucleotide sequence encoding an antigenic polypeptide.

Recombinant and DNA-type immunogenic compositions and vaccines may comprise an adjuvant.

In the context of the combined immunization or vaccination programmes, it is also possible to combine the immunization or vaccination against the porcine circovirus and the porcine parvovirus with an immunization or vaccination against other pig pathogens, in particular those which could be associated with the PMWS syndrome. The immunogenic composition or vaccine according to the invention may therefore comprise another valency corresponding to another pig pathogen chosen from PRRS (Porcine Reproductory and Respiratory Syndrome) and/or Mycoplasma hyopneumoniae, and/or E. coli, and/or Atrophic Rhinitis, and/or Pseudorabies (Aujeszky's disease) virus and/or porcine influenza and/or Actinobacillus pleuropneumoniae and/or Hog cholera, and

combinations thereof. Preferably, the programme of immunization or vaccination and the vaccines according to the invention will combine immunizations or vaccinations against the circovirus and the parvovirus, and the PRRS (WO-A-93/07898, WO-A-94/18311, FR-A-2 709 966 ; C. Charreyre et al., Proceedings of the 15th IPVS Congress, Birmingham, England, 5-9 July 1998, p 139 ; and/or Mycoplasma hyopneumoniae (EP-A-597 852, EP-A-550 477, EP-A571 648 ; O. Martinon et al. p 157, 284, 285 and G. Reynaud et al., p 150, all in the above-referenced Proceedings of the 15th IPVS Congress) and/or porcine influenza. It is thus possible to use any appropriate form of immunogenic composition or vaccine, in particular any available commercial vaccine, so as to combine it with the immunogenic composition or vaccine against the porcine circovirus and porcine parvovirus as described here.

The subject of the present invention is therefore also multivalent immunogenic compositions and vaccines, multivaccine kits, and combined immunization or vaccination methods which make it possible to use such combined immunization or vaccination programmes.

The invention will now be described in greater detail with the aid of nonlimiting exemplary embodiments, taken with reference to the drawing, in which:

Figure 1: DNA sequence of the genome of the Imp. 1011-48121 strain

Figure 2: DNA sequence of the genome of the Imp. 1011-48285 strain

Figure 3: DNA sequence of the genome of the Imp. 999 strain

Figure 4: DNA sequence of the genome of the Imp. 1010 strain

Figure 5: Alignment of the 4 sequences according to Figures 1 to 4 with the sequence of the PCV PK/15 strain

Sequence listing SEQ ID

SEQ ID No: 1 DNA sequence of the genome of the Imp. 1011-48121 strain

SEQ ID No: 2 DNA sequence of the genome of the Imp. 1011-48285 strain

SEQ ID No: 3 DNA sequence of the genome of the Imp. 999 strain

SEQ ID No: 4 DNA sequence of the genome of the Imp. 1010 strain

SEQ ID No: 5 DNA sequence of the genome of the PK/15 strain

EXAMPLES

Example 1: Culture and isolation of the porcine circovirus strains:

Tissue samples were collected in France, Canada and the USA from lung and lymph nodes of piglets. These piglets exhibited clinical signs typical of the post-weaning multisystemic wasting syndrome. To facilitate the isolation of the viruses, the tissue samples were frozen at -70°C immediately after autopsy.

For the viral isolation, suspensions containing about 15% tissue sample were prepared in a minimum medium containing Earle's salts (EMEM, BioWhittaker UK Ltd., Wokingham, UK), penicillin (100 IU/ml) and streptomycin (100 µg/ml) (MEM-SA medium), by grinding tissues with sterile sand using a sterile mortar and pestle. This ground preparation was then taken up in MEM-SA, and then centrifuged at 3000 g for 30 minutes at +4°C in order to harvest the supernatant.

Prior to the inoculation of the cell cultures, a volume of 100 µl of chloroform was added to 2 ml of each supernatant and mixed continuously for 10 minutes at room temperature. This mixture was then transferred to a microcentrifuge tube, centrifuged at 3000 g for 10 minutes, and then the supernatant was harvested. This supernatant was then used as inoculum for the viral isolation experiments.

All the viral isolation studies were carried out on PK/15 cell cultures, known to be uncontaminated with the porcine circovirus (PCV), pestiviruses, porcine adenoviruses and porcine parvoviruses (Allan G. *et al* Pathogenesis of porcine circovirus experimental infections of colostrum-deprived piglets and examination of pig foetal material. Vet. Microbiol. 1995, 44, 49-64).

The isolation of the porcine circoviruses was carried out according to the following technique:

Monolayers of PK/15 cells were dissociated by trypsinization (with a trypsin-versene mixture) from confluent cultures, and taken up in MEM-SA medium containing 15% foetal calf serum not contaminated by pestivirus (= MEM-G medium) in a final concentration of about 400,000 cells per ml. 10 ml aliquot fractions of this cell suspension were then mixed with 2 ml aliquot fractions of the inocula described above, and the final mixtures were aliquoted in 6 ml volumes in two Falcon flasks of 25 cm^2. These cultures were then incubated at +37°C for 18 hours under an atmosphere containing 10% CO_2.

After incubation, the culture medium of the semi-confluent monolayers were treated with 300 mM D-glucosamine (Cat # G48175, Sigma-Aldrich Company Limited, Poole, UK) (Tischr I. et al., Arch. Virol., 1987 **96** 39-57), then incubation was continued for an additional period of 48-72 hours at +37°C. Following this last incubation, one of the two Falcons of each inoculum was subjected to 3 successive freeze/thaw cycles. The PK/15 cells of the remaining Falcon were treated with a trypsin-versene solution, resuspended in 20 ml of MEM-G medium, and then inoculated into 75 cm^2 Falcons at a concentration of 400,000 cells/ml. The freshly inoculated flasks were then "superinfected" by addition of 5 ml of the corresponding lysate obtained after the freeze/thaw cycles.

Example 2: Preparation of the samples of cell culture for the detection of porcine circoviruses by immunofluorescence or by *in situ* hybridization

A volume of 5 ml of the "superinfected" suspension was collected and inoculated into a Petri dish 55 mm in diameter containing a sterile and fat-free glass coverslip. The cultures in the flasks and on glass coverslips were incubated at +37°C and treated with glucosamine as described in Example 1. The cultures on glass coverslips were harvested from 24 to 48 hours after the treatment with glucosamine and fixed, either with acetone for 10 minutes at room temperature, or with 10% buffered formaldehyde for 4 hours. Following this fixing, all the glass coverslips were stored at -70°C, on silica gel, before their use for the *in situ* hybridization studies and the immunocytochemical labelling studies.

Example 3: Techniques for the detection of PCV sequences by *in situ* hybridization

In situ hybridization was carried out on tissues collected from diseased pigs and fixed with formaldehyde and also on the preparations of cell cultures inoculated for the viral isolation (see Example 2) and fixed on glass coverslips.

Complete genomic probes corresponding to the PK/15 porcine circoviruses (PCV) and to the infectious chicken anaemia virus (CAV) were used. The plasmid pPCV1, containing the replicative form of the PCV genome, cloned in the form of a single 1.7 kilo base pair (kbp) insert (Meehan B. *et al.* Sequence of porcine circovirus DNA: affinities with plant circoviruses, J. Gen. Virol. 1997, **78**, 221-227), was used as specific viral DNA source for PCV. An analogous plasmid, pCAA1, containing the 2.3 kbp replicative form of the avian circovirus CAV was used as negative control. The respective glycerol stocks of the two plasmids were used for the production and purification of the plasmids according to the alkaline lysis technique

(Sambrook J. *et al.* Molecular cloning: A Laboratory Manual. 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989) so that they are then used as templates for the preparation of the probes. The circovirus probes representative of the complete genomes of PCV and of CAV were produced from the purified plasmids described above (1 µg for each probe) and from hexanucleotide primers at random using a commercial nonradioactive labelling kit ("DIG DNA labelling kit", Boehringer Mannheim, Lewes, UK) according to the supplier's recommendations.

The digoxigenin-labelled probes were taken up in a volume of 50-100 µl of sterile water before being used for the *in situ* hybridization.

The diseased pig tissue samples, enclosed in paraffin and fixed with formaldehyde, as well as the preparations of infected cell cultures, fixed with formaldehyde, were prepared for the detection of the PCV nucleic acids according to the following technique:

Sections 5 µm thick were cut from tissue blocks enclosed in paraffin, rendered paraffin free, and then rehydrated in successive solutions of alcohol in decreasing concentrations. The tissue sections and the cell cultures fixed with formaldehyde were incubated for 15 minutes and 5 minutes respectively at +37°C in a 0.5% proteinase K solution in 0.05 M Tris-HCl buffer containing 5 mM EDTA (pH 7.6). The slides were then placed in a 1% glycine solution in autoclaved distilled water, for 30 seconds, washed twice with 0.01 M PBS buffer (phosphate buffered saline) (pH 7.2), and finally washed for 5 minutes in sterile distilled water. They were finally dried in the open air and placed in contact with the probes.

Each tissue/probe preparation was covered with a clean and fat-free glass coverslip, and then placed in an oven at +90°C for 10 minutes, and then placed in contact with an ice block for 1 minute, and finally incubated for 18 hours at +37°C. The preparations were then briefly immersed in a 2X sodium citrate salt (SSC)

buffer (pH 7.0) in order to remove the protective glass coverslips, and then washed twice for 5 minutes in 2X SSC buffer and finally washed twice for 5 minutes in PBS buffer.

After these washes, the preparations were immersed in a solution of 0.1 M maleic acid, 0.15 M NaCl (pH 7.5) (maleic buffer) for 10 minutes, and then incubated in a 1% solution of blocking reagent (Cat # 1096176, Boehringer Mannheim UK, Lewis, East Sussex, UK) in maleic buffer for 20 minutes at +37°C.

The preparations were then incubated with a 1/250 solution of an anti-digoxigenin monoclonal antibody (Boehringer Mannheim), diluted in blocking buffer, for 1 hour at +37°C, washed in PBS and finally incubated with a biotinylated anti-mouse immunoglobulin antibody for 30 minutes at +37°C. The preparations were washed in PBS and the endogenous peroxidase activity was blocked by treatment with a 0.5% hydrogen peroxide solution in PBS for 20 minutes at room temperature. The preparations were again washed in PBS and treated with a 3-amino-9-diethylcarbazole (AEC) substrate (Cambridge Bioscience, Cambridge, UK) prepared immediately before use.

After a final wash with tap water, the preparations were counterstained with hematoxylin, "blued" under tap water, and mounted on microscope glass coverslips with a mounting fluid (GVA Mount, Cambridge Bioscience, Cambridge, UK). The experimental controls included the use of a nonpertinent negative probe (CAV) and of a positive probe (PCV) on samples obtained from diseased pigs and from nondiseased pigs.

Example 4: Technique for the detection of PCV by immunofluorescence

The initial screening of all the cell culture preparations fixed with acetone was carried out by an indirect immunofluorescence technique (IIF) using a 1/100 dilution of a pool of adult pig sera. This pool of sera comprises sera from 25 adult sows from Northern

Ireland and is known to contain antibodies against a wide variety of porcine viruses, including PCV: porcine parvovirus, porcine adenovirus, and PRRS virus. The IIF technique was carried out by bringing the serum (diluted in PBS) into contact with the cell cultures for one hour at +37°C, followed by two washes in PBS. The cell cultures were then stained with a 1/80 dilution in PBS of a rabbit anti-pig immunoglobulin antibody conjugated with fluorescein isothiocyanate for one hour, and then washed with PBS and mounted in glycerol buffer prior to the microscopic observation under ultraviolet light.

Example 5: Results of the *in situ* hybridization on diseased pig tissues

The *in situ* hybridization, using a PCV genomic probe, prepared from tissues collected from French, Canadian and Californian piglets having multisystemic wasting lesions and fixed with formaldehyde, showed the presence of PCV nucleic acids associated with the lesions, in several of the lesions studied. No signal was observed when the PCV genomic probe was used on tissues collected from nondiseased pigs or when the CAV probe was used on the diseased pig tissues. The presence of PCV nucleic acid was identified in the cytoplasm and the nucleus of numerous mononuclear cells infiltrating the lesions in the lungs of the Californian piglets. The presence of PCV nucleic acid was also demonstrated in the pneumocytes, the bronchial and bronchiolar epithelial cells, and in the endothelial cells of the arterioles, the veinlets and lymphatic vessels.

In diseased French pigs, the presence of PCV nucleic acid was detected in the cytoplasm of numerous follicular lymphocytes and in the intrasinusoidal mononuclear cells of the lymph nodes. The PCV nucleic acid was also detected in occasional syncytia. Depending on these detection results, samples of Californian pig lungs, French pig mesenteric lymph

nodes, and Canadian pig organs were selected for the purpose of isolating new porcine circovirus strains.

Example 6: Results of the cell culture of the new porcine circovirus strains and detection by immunofluorescence

No cytopathic effect (CPE) was observed in the cell cultures inoculated with the samples collected from French piglets (Imp.1008 strain), Californian piglets (Imp.999 strain) and Canadian piglets (Imp.1010 strain) showing clinical signs of multisystemic wasting syndrome. However, immunolabelling of the preparations obtained from the inoculated cell cultures, after fixing using acetone and with a pool of pig polyclonal sera, revealed nuclear fluorescence in numerous cells in the cultures inoculated using the lungs of Californian piglets (Imp.999 strain), using the mediastinal lymph nodes of French piglets (Imp.1008 strain), and using organs of Canadian piglets (Imp.1010 strain).

Example 7: Extraction of the genomic DNA of the porcine circoviruses

The replicative forms of the new strains of porcine circoviruses (PCV) were prepared using infected PK/15 cell cultures (see Example 1) (10 Falcons of 75 cm^2) harvested after 72-76 hours of incubation and treated with glucosamine, as described for the cloning of the replicative form of CAV (Todd. D. *et al.* Dot blot hybridization assay for chicken anaemia agent using a cloned DNA probe. J. Clin. Microbiol. 1991, **29**, 933-939). The double-stranded DNA of these replicative forms was extracted according to a modification of the Hirt technique (Hirt B. Selective extraction of polyoma virus DNA from infected cell cultures, J. Mol. Biol. 1967, **36**, 365-369), as described by Molitor (Molitor T.W. *et al.* Porcine parvovirus DNA: characterization of the genomic and replicative form DNA of two virus isolates, Virology, 1984, **137**, 241-254).

Example 8: Restriction map of the replicative form of the genome of the porcine circovirus Imp.999 strain.

The DNA (1-5 µg) extracted according to the Hirt technique was treated with S1 nuclease (Amersham) according to the supplier's recommendations, and then this DNA was digested with various restriction enzymes (Boehringer Mannheim, Lewis, East Sussex, UK) and the products of digestion were separated by electrophoresis on a 1.5% agarose gel in the presence of ethidium bromide as described by Todd *et al.* (Purification and biochemical characterization of chicken anemia agent. J. Gen. Virol. 1990, **71**, 819-823). The DNA extracted from the cultures of the Imp.999 strain possess a unique EcoRI site, 2 SacI sites and do not possess any PstI site. This restriction profile is therefore different from the restriction profile shown by the PCV PK/15 strain (Meehan B. *et al.* Sequence of porcine circovirus DNA; affinities with plant circoviruses, 1997 **78**, 221-227) which possess in contrast a PstI site and do not possess any EcoRI site.

Example 9: Cloning of the genome of the porcine circovirus Imp.999 strain

The restriction fragment of about 1.8 kbp generated by digestion of the double-stranded replicative form of the PCV Imp.999 strain with the restriction enzyme EcoRI was isolated after electrophoresis on a 1.5% agarose gel (see Example 3) using a Qiagen commercial kit (QIAEXII Gel Extraction Kit, Cat # 20021, QIAGEN Ltd., Crawley, West Sussex, UK). This EcoRI-EcoRI restriction fragment was then ligated with the vector pGEM-7 (Promega, Medical Supply Company, Dublin, Ireland), previously digested with the same restriction enzymes and dephosphorylated, according to standard cloning techniques (Sambrook J. *et al.* Molecular cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). The plasmids obtained were

transformed into an *Escherichia coli* JM109 host strain (Stratagene, La Jolla, USA) according to standard techniques. The EcoRI-EcoRI restriction fragment of the PCV Imp.999 strain was also cloned into the EcoRI site of the vector pBlueScript SK+ (Stratagene Inc. La Jolla, USA). Among the clones obtained for each host strain, at least 2 clones containing the fragments of the expected size were selected. The clones obtained were then cultured and the plasmids containing the complete genome of the Imp.999 strain were purified in a small volume (2 ml) or in a large volume (250 ml) according to standard plasmid preparation and purification techniques.

Example 10: Sequencing of a genomic DNA (double-stranded replicative form) of the PCV Imp.999 strain.

The nucleotide sequence of 2 EcoRI Imp.999 clones (clones pGEM-7/2 and pGEM-7/8) was determined according to Sanger's dideoxynucleotide technique using the sequencing kit "AmpliTaq DNA polymerase FS" (Cat # 402079 PE Applied Biosystems, Warrington, UK) and an Applied BioSystems AB1373A automatic sequencing apparatus according to the supplier's recommendations. The initial sequencing reactions were carried out with the M13 "forward" and "reverse" universal primers. The following sequencing reactions were generated according to the "DNA walking" technique. The oligonucleotides necessary for these subsequent sequencings were synthesized by Life Technologies (Inchinnan Business Park, Paisley, UK).

The sequences generated were assembled and analysed by means of the MacDNASIS version 3.2 software (Cat # 22020101, Appligene, Durham, UK). The various open reading frames were analysed by means of the BLAST algorithm available on the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA) server.

The complete sequence (EcoRI-EcoRI fragment) is presented in SEQ ID No: 3 (Figure 3). It gives the

total sequence of this strain, which was made to start arbitrarily at the beginning of the EcoRI site, that is to say the G as the first nucleotide.

The procedure was carried out in a similar manner for obtaining the sequence of the other three isolates according to the invention (see SEQ ID No: 1, 2 and 4 and Figures 1, 2 and 4).

The size of the genome of these four strains is:

Imp. 1011-48121	1767 nucleotides
Imp. 1011-48285	1767 nucleotides
Imp. 999	1768 nucleotides
Imp. 1010	1768 nucleotides

Example 11: Analysis of the sequence of the PCV Imp.999 strain.

When the sequence generated from the Imp.999 strain was used to test for homology with respect to the sequences contained in the GenBank databank, the only significant homology which was detected is a homology of about 76% (at nucleic acid level) with the sequence of the PK/15 strain (accession numbers Y09921 and U49186) (see Figure No. 5).

At amino acid level, the test for homology in the translation of the sequences in the 6 phases with the databanks (BLAST X algorithm on the NABI server) made it possible to demonstrate a 94% homology with the open reading frame corresponding to the theoretical replicase of the BBTV virus similar to the circoviruses of plants (GenBank identification number 1841515) encoded by the GenBank U49186 sequence.

No other sequence contained in the databanks show significant homology with the sequence generated from the PCV Imp.999 strain.

Analysis of the sequences obtained from the Imp.999 strain cultured using lesions collected from Californian piglets having clinical signs of the multisystemic wasting syndrome shows clearly that this viral isolate is a new porcine circovirus strain.

Example 12: Comparative analysis of the sequences

The alignment of the nucleotide sequences of the 4 new PCV strains was made with the sequence of the PCV PK/15 strain (Figure 5). A homology matrix taking into account the four new strains and the previous PK/15 strain was established. The results are the following:

1 : Imp. 1011-48121
2 : Imp. 1011-48285
3 : Imp. 999
4 : Imp. 1010
5: PK/15

	1	2	3	4	5
1	1.0000	0.9977	0.9615	0.9621	0.7600
2		1.0000	0.9621	0.9632	0.7594
3			1.0000	0.9949	0.7560
4				1.0000	0.7566
5					1.0000

The homology between the two French strains Imp. 1011-48121 and Imp. 1011-48285 is greater than 99% (0.9977).

The homology between the two North American strains Imp. 999 and Imp. 1010 is also greater than 99% (0.9949). The homology between the French strains and the North American strains is slightly greater than 96%.

The homology between all these strains and PK/15 falls at a value between 75 and 76%.

It is deduced therefrom that the strains according to the invention are representative of a new type of porcine circovirus, distinct from the type represented by the PK/15 strain. This new type, isolated from pigs exhibiting the PMWS syndrome, is called type II porcine circovirus, PK/15 representing type I. The strains belonging to this type II exhibit remarkable nucleotide sequence homogeneity, although

they have in fact been isolated from very distant geographical regions.

Example 13: Analysis of the proteins encoded by the genome of the new PCV strains.

The nucleotide sequence of the Imp. 1010 isolate was considered to be representative of the other circovirus strains associated with the multi-systemic wasting syndrome. This sequence was analysed in greater detail with the aid of the BLASTX algorithm (Altschul *et al.* J. Mol. Biol. 1990. **215**. 403-410) and of a combination of programs from the set of MacVector 6.0 software (Oxford Molecular Group, Oxford OX4 4GA, UK). It was possible to detect 13 open reading frames (or ORFs) of a size greater than 20 amino acids on this sequence (circular genome). These 13 ORFs are the following:

Name	Start	End	Strand	Size of the ORF (nucleotides (nt))	Protein size (amino acids (aa))
ORF1	103	210	sense	108 nt	35 aa
ORF2	1180	1317	sense	138 nt	45 aa
ORF3	1363	1524	sense	162 nt	53 aa
ORF4	398	1342	sense	945 nt	314 aa
ORF5	900	1079	sense	180 nt	59 aa
ORF6	1254	1334	sense	81 nt	26 aa
ORF7	1018	704	antisense	315 nt	104 aa
ORF8	439	311	antisense	129 nt	42 aa
ORF9	190	101	antisense	90 nt	29 aa
ORF10	912	733	antisense	180 nt	59 aa
ORF11	645	565	antisense	81 nt	26 aa
ORF12	1100	1035	antisense	66 nt	21 aa
ORF13	314	1381	antisense	702 nt	213 aa

The positions of the start and end of each ORF refer to the sequence presented in Figure No. 4 (SEQ ID No. 4), of the genome of strain 1010. The

limits of ORFs 1 to 13 are identical for strain 999. They are also identical for strains 1011-48121 and 1011-48285, except for the ORFs 3 and 13:

ORF3 1432-1539, sense, 108 nt, 35aa

ORF13 314-1377, antisense, 705 nt, 234 aa.

Among these 13 ORFs, 4 have a significant homology with analogous ORFs situated on the genome of the cloned virus PCV PK-15. Each of the open reading frames present on the genome of all the circovirus isolates associated with the multisystemic wasting syndrome was analysed. These 4 ORFs are the following:

Name	Start	End	Strand	Size of the ORF (nt)	Protein size (aa)	Molecular mass
ORF4	398	1342	sense	945 nt	314 aa	37.7 kDa
ORF7	1018	704	antisense	315 nt	104 aa	11.8 kDa
ORF10	912	733	antisense	180 nt	59 aa	6.5 kDa
ORF13	314	1381	antisense	702 nt	233 aa	27.8 kDa

The positions of the start and end of each ORF refer to the sequence presented in Figure No. 4 (SEQ ID No. 4). The size of the ORF (in nucleotides = nt) includes the stop codon.

The comparison between the genomic organization of the PCV Imp. 1010 and PCV PK-15 isolates allowed the identification of 4 ORFs preserved in the genome of the two viruses. The table below presents the degrees of homology observed:

ORF Imp. 1010/ORF PVC PK-15	**Percentage homology**
ORF4/ORF1	86%
ORF13/ORF2	66.4%
ORF7/ORF3	61.5% (at the level of the overlap (104 aa))
ORF10/ORF4	83% (at the level of the overlap (59 aa))

The greatest sequence identity was observed between ORF4 Imp. 1010 and ORF1 PK-15 (86% homology). This was expected since this protein is probably involved in the replication of the viral DNA and is essential for the viral replication (Meehan *et al.* J. Gen. Virol. 1997. **78**. 221-227; Mankertz *et al.* J. Gen. Virol. 1998. **79**. 381-384).

The sequence identity between ORF13 Imp. 1010 and ORF2 PK-15 is less strong (66.4% homology), but each of these two ORFs indeed exhibits a highly conserved N-terminal basic region which is identical to the N-terminal region of the major structural protein of the CAV avian circovirus (Meehan *et al.* Arch. Virol. 1992. **124**. 301-319). Furthermore, large differences are observed between ORF7 Imp. 1010 and ORF3 PK-15 and between ORF10 Imp. 1010 and ORF4 PK-15. In each case, there is a deletion of the C-terminal region of the ORF7 and ORF10 of the Imp. 1010 isolate when they are compared with ORF3 and ORF4 of PCV PK-15. The greatest sequence homology is observed at the level of the N-terminal regions of ORF7/ORF3 (61.5% homology at the level of the overlap) and of ORF10/ORF4 (83% homology at the level of the overlap).

It appears that the genomic organization of the porcine circovirus is quite complex as a consequence of the extreme compactness of its genome. The major structural protein is probably derived from splicing between several reading frames situated on the same strand of the porcine circovirus genome. It can therefore be considered that any open reading frame (ORF1 to ORF13) as described in the table above can represent all or part of an antigenic protein encoded by the type II porcine circovirus and is therefore potentially an antigen which can be used for specific diagnosis and/or for vaccination. The invention therefore relates to any protein comprising at least one of these ORFs. Preferably, the invention relates to a protein essentially consisting of ORF4, ORF7, ORF10 or ORF13.

Example 14: Infectious character of the PCV genome cloned from the new strains.

The plasmid pGEM-7/8 containing the complete genome (replicative form) of the Imp.999 isolate was transfected into PK/15 cells according to the technique described by Meehan B. *et al.* (Characterization of viral DNAs from cells infected with chicken anemia agent: sequence analysis of the cloned replicative form and transfection capabilities of cloned genome fragments. Arch. Virol. 1992, **124**, 301-319). Immunofluorescence analysis (see Example 4) carried out on the first passage after transfection on noncontaminated PK/15 cells have shown that the plasmid of the clone pGEM7/8 was capable of inducing the production of infectious PCV virus. The availability of a clone containing an infectious PCV genetic material allows any useful manipulation on the viral genome in order to produce modified PCV viruses (either attenuated in pigs, or defective) which can be used for the production of attenuated or recombinant vaccines, or for the production of antigens for diagnostic kits.

Example 15: Production of PCV antigens by *in vitro* culture

The culture of the noncontaminated PK/15 cells and the viral multiplication were carried out according to the same methods as in Example 1. The infected cells are harvested after trypsinization after 4 days of incubation at 37°C and enumerated. The next passage is inoculated with 400,000 infected cells per ml.

Example 16: Inactivation of the viral antigens

At the end of the viral culture, the infected cells are harvested and lysed using ultrasound (Branson Sonifier) or with the aid of a rotor-stator type colloid mill (UltraTurrax, IKA). The suspension is then centrifuged at 3700 g for 30 minutes. The viral suspension is inactivated with 0.1% ethyleneimine for

18 hours at +37°C or with 0.5% beta-propiolactone for 24 hours at +28°C. If the virus titre before inactivation is inadequate, the viral suspension is concentrated by ultrafiltration using a membrane with a 300 kDa cut-off (Millipore PTMK300). The inactivated viral suspension is stored at +5°C.

Example 17: Preparation of the vaccine in the form of an emulsion based on mineral oil.

The vaccine is prepared according to the following formula:

- suspension of inactivated porcine circovirus: 250 ml
- Montanide® ISA 70 (SEPPIC): 750 ml

The aqueous phase and the oily phase are sterilized separately by filtration. The emulsion is prepared by mixing and homogenizing the ingredients with the aid of a Silverson turbine emulsifier.

One vaccine dose contains about $10^{7.5}$ TCID50. The volume of one vaccine dose is 0.5 ml for administration by the intradermal route, and 2 ml for administration by the intramuscular route.

This vaccine is used in a vaccination programme against the multisystemic wasting syndrome in combination with the Parvovax® vaccine.

Example 18: Preparation of the vaccine in the form of a metabolizable oil-based emulsion.

The vaccine is prepared according to the following formula:

- suspension of inactivated porcine circovirus: 200 ml
- Dehymuls HRE 7 (Henkel): 60 ml
- Radia 7204 (Oleofina): 740 ml

The aqueous phase and the oily phase are sterilized separately by filtration. The emulsion is prepared by mixing and homogenizing the ingredients with the aid of a Silverson turbine emulsifier.

One vaccine dose contains about $10^{7.5}$ TCID50. The volume of one vaccine dose is 2 ml for administration by the intramuscular route.

This vaccine is used in a vaccination programme against the multisystemic wasting syndrome in combination with the Parvovax® vaccine.

Example 19: The indirect immunofluorescence results in relation to the US and French PCV virus strains and to the PK/15 contaminant with a hyperimmune serum (PCV-T), a panel of monoclonal antibodies F99 prepared from PK/15 and a hyperimmune serum prepared from the Canadian strain (PCV-C)

VIRUS			
	PK/15	USA	France
PCV-T antiserum	≥ 6400	200	800
PCV-C antiserum	200	≥ 6.400	≥ 6.400
F99 1H4	≥ 10 000	<100	100
F99 4B10	≥ 10 000	<100	<100
F99 2B7	≥ 10 000	100	<100
F99 2E12	≥ 10 000	<100	<100
F99 1C9	≥ 10 000	<100	100
F99 2E1	≥ 10 000	<100	<100
F99 1H4	≥ 10 000	100	<100

* Reciprocal of the last dilution of the serum or of the monoclonal antibody which gives a positive reaction in indirect immunofluorescence.

Example 20: Experimental production of the porcine multisystemic wasting syndrome - protocol 1

Three-day old gnotobiotic piglets obtained by caesarean and kept in an isolating unit were inoculated with virus solutions of PCV. The type II PCV viruses used were the Imp 1010 isolate and the virus obtained from lymph node homogenates obtained from diseased pigs.

Five groups were formed. The piglets were all inoculated at the age of three days by the oronasal route with 1.5 ml of virus solution according to the following scheme:

Group	Number	Virus	Dose
A	6	Lymph node homogenate	ND
B	5	Imp. 1010 (low passage)	10^2 TCID50
C	4	Imp. 1010 (high passage)	10^2 TCID50
D	2	Lysate of PK15 cells free of PCV virus	---
E	3	---	---

Results of the experimental challenge:

During the 5-week observation period, the piglets did not develop clinical signs, apart from one animal in group B which showed substantial exhaustion. At autopsy, the pigs in groups A, B and C exhibit hyperplasia of the lymph nodes (size 2 to 10 times greater than that for the animals in groups D and E), in particular of the submaxillary, bronchial, mesenteric, iliac and femoral ganglia. This hyperplasia is linked to a considerable expansion of the cortical zones by infiltration by monocytes and macrophages.

The piglets in groups A, B and C also exhibit hyperplasia of the bronchial lymphoid tissue.

One piglet in each of groups A, B and C has pneumonia.

The piglet in group B, which exhibited substantial exhaustion, and one piglet in group A have a gastric ulcer.

Moreover, all the animals in groups A, B and C have myositis in the muscular tunica of the stomach and of the intestine.

Most of the animals in groups A, B and C have myocarditis, multifocal hepatitis with lymphocyte, macrophage and eosinophile infiltration, as well as cortical and medullary interstitial nephritis.

One piglet in group C has a liver whose size is bigger than normal, with disseminated clear foci at its surface.

No lesion was observed in the piglets in groups D and E.

Circovirus was isolated from the organs of pigs in groups A, B and C.

Example 21: Experimental reproduction of the porcine multisystemic wasting syndrome - protocols 2 and 3

Conventional piglets, but isolated from their mother from birth, were inoculated with viral solutions of type II PCV, of porcine parvovirus, or with a mixture of the two viruses.

The type II PCV viruses used were the Imp. 1010 and Imp. 1011 isolates (strain 48121).

The PPV virus used is an isolate of Canadian origin, Imp. 1005. This virus has a sequence (1/3 of the sequenced genome) which is identical to that of other known porcine parvovirus strains (PPV strain NADL-2 and Kresse strain).

Two experimental protocols were carried out.

Protocol 2

Three groups were formed with 3-day-old piglets. The piglets were all inoculated by the oronasal route with 1 ml of viral solution according to the following scheme:

Group	Number	Virus	Dose
A	5	Imp. 1010	10^7 TCID50
B	5	Imp. 1010 + Imp. 1005	5×10^6 TCID50
C (control)	2	---	---

Results of the experimental challenge:

Group A: 2 piglets died 21 days after the inoculation and one piglet was humanely killed 24 days after the inoculation.

Group B: 1 piglet died 23 days after the inoculation and one piglet was humanely killed 24 days after the inoculation.

The autopsies carried out on the piglets that died following an infection showed the presence of substantial macroscopic lesions: presence of fluid in the pleural cavity, lung oedema, haemorrhages in the kidneys, whitish lesions in the form of a pin head on the kidneys, hepatic necrosis. These lesions are identical to those observed in the field cases.

The autopsies carried out on the sacrificed piglets did not show macroscopic lesions.

The histological examinations performed on organs removed from the piglets in groups A and B which died following an infection, as well as in the sacrificed pigs in these 2 groups, showed a typical and complete pattern of the lesions of porcine multisystemic wasting syndrome which are observed in animals in the field: hepatic necrosis, necrosis of the lymph nodes, pancreatic necrosis, focal necrosis and severe haemorrhages in the kidneys, presence of syncytia in the lungs, severe necrosis of the hepatocytes with the presence of nuclear inclusions.

It should be noted that a massive quantity of PCV antigen was found in all these lesions (dead or sacrificed pigs), but that the presence of PPV antigen could not be detected in these same lesions.

No lesion could be detected in the control piglets in group C.

Protocol 3

Four groups were formed with 4-week-old piglets. The pigs were all inoculated by the oronasal route with 1 ml of viral solution according to the following scheme:

Group	Number	Virus	Dose
A (control)	2	---	---
B	4	Imp. 1005 (PPV)	$10^{5.3}$ TCID50
C	4	Imp. 1011 (PCV)	10^{5} TCID50
D	4	Imp. 1005 + Imp. 1011	$10^{5}+5\times10^{4}$ TCID50

Results of the experimental challenge:

1 "control" piglet and 2 piglets in each experimental group (B, C and D) were humanely killed and subjected to autopsy 2 weeks after inoculation. Significant immunohistological lesions were observed in the two piglets in group D (PCV + PPV coinfection). It should be noted that it was not possible to detect the presence of porcine parvovirus in these lesions, although a seroconversion in relation to the porcine parvovirus was observed in all the pigs in group D.

No macroscopic or histological lesion could be observed in the control piglet and in the piglets in the other groups.

It therefore appears that the PCV + PPV combination makes it possible to reproduce histological lesions typical of the porcine multisystemic wasting syndrome.

Following these two experimental protocols, it can be observed that the inoculation of PCV alone, as a PCV + PPV mixture, leads to a more or less severe reproduction of the porcine multisystemic wasting syndrome, but only the porcine circovirus can be detected in the lesions. By contrast, an experimental infection with PPV alone (group B of protocol 3) does not allow macroscopic or histological lesions to be induced; however, in the presence of PCV, the appearance of lesions is observed in 4-week-old pigs (group D of protocol 3).